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Do environmental factors affect male fathead minnow (*Pimephales promelas*) response to estrone? Part 1. Dissolved oxygen and sodium chloride

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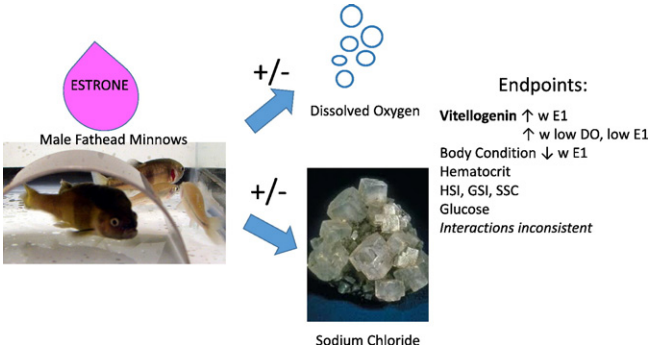
HIGHLIGHTS

- Cause for disparity between lab and field effects of estrogens on fish unknown
- Investigated impact of DO_2 and NaCl concentration on fish response to estrone.
- At low estrone and low DO_2, respiration and vitellogenin up
- NaCl and DO_2 concentrations tested did not consistently alter estrone response.
- Future work should include lower DO_2 and higher NaCl concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

Laboratory exposures indicate that estrogens and their mimics can cause endocrine disruption in male fishes, yet while studies of resident fish populations in estrogen-polluted waters support these findings, biomarker expression associated with field versus laboratory exposure to estrogenic endocrine disruptors (EDs) often differ dramatically. Two of the environmental parameters often found to vary in dynamic aquatic ecosystems were chosen (dissolved oxygen [DO] and sodium chloride concentrations) to assess their potential impact on ED exposure. In separate experiments, male fathead minnows (*Pimephales promelas*) were exposed to estrone (E1) a natural ED, under either two concentrations of DO, or two concentrations of sodium chloride, in a laboratory flow-through system. Morphological and hematological parameters were assessed. While vitellogenin concentrations were elevated with exposure to estrone (29 to 390 ng/L), the effect on other indices were variable. Estrone exposure altered SSC, blood glucose, hematocrit, and hepatic and gonado-somatic index in 1 of 4 experiments, while it decreased body condition factor in 3 of 4 experiments. At the concentrations tested, no main effect differences ($P < 0.05$) were found associated with DO or sodium chloride treatments, except in one experiment low DO resulted in a decrease in secondary sex characteristic score (SSC). The combination of DO or sodium chloride and E1 altered blood glucose in one experiment each. These results indicate the variability of fathead minnow response to estrone, even within the confines of controlled laboratory conditions.

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1. Introduction

1.1. Estrogens in a dynamic environment

Anthropogenic influences, such as agricultural practices (Chen et al., 2010; Kjær et al., 2007; Mattheissen et al., 2006) and urban runoff (Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al., 2013), place a chemical burden on aquatic ecosystems. Estrogenic compounds, such as estrone (E1), 17β-estradiol (E2), and the synthetic 17α-ethynylestradiol (EE2) can be found in anthropogenically influenced aquatic ecosystems (Kolpin et al., 2002; Lee et al., 2011) and interfere with reproductive function in fish (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Schultz et al., 2013; Vajda et al., 2008). While examination of concentration-dependent effects of individual aquatic contaminants is relatively uncomplicated due to standardized culture techniques (Denny, 1987) and exposure protocols (EPA (Environmental Protection Agency), 2002), there remains a dearth of information on how physico-chemical characteristics of aquatic environments modulate endocrine response to estrogenic contaminants of emerging concern. Closing this knowledge gap is urgent as the ecological reach of human impact broadens (Oreskes, 2004).

1.2. Hypoxia

Historical data (Stahl et al., 2013) and models of freshwater systems (Jeppesen et al., 2010; Moore et al., 1997) suggest an increasing prevalence of hypoxic conditions as a result of climate change, due to increased surface water temperature (Murdoch et al., 2000), prolonged thermal stratification (Taner et al., 2011) and increased nutrient runoff often associated with agricultural land use (Adrian et al., 2009). Aquatic ecosystems impacted by these factors are often the same areas associated with the discharge of estrogenic contaminants of emerging concern (Barber et al., 2011; Chen et al., 2010; Kjær et al., 2007; Lee et al., 2011; Martinovic-Weigelt et al., 2013; Mattheissen et al., 2006). Hypoxic conditions suppress reproductive function in fish (*Fundulus grandis*, Landry et al., 2007; *Micropogonias undulatus;* Thomas et al., 2006; and *Cyprinus carpio*, Wu et al., 2003), and increase estrogen receptor transcriptional activity synergistically in combination with E2 (Yi et al., 2009).

It is unclear just why hypoxic conditions alter endocrine function in fish. In part, the altered response may reflect behavioral changes that result in higher exposures to effluent. Spigarelli et al. (1982) reported an increased affinity of fish to congregate near discharges in which water temperature was 1–3 °C greater than the maximum ambient water temperature. Two environmental conditions typically occur at these sites, increased water temperature and decreased oxygen concentrations. Fish would respond to both factors with an increased respiratory rate (Gee et al., 1978; Wares and Igram, 1979). As the lipophilic gill tissues of teleost fish are thought to be a primary site for uptake of steroidal compounds (Blewett et al., 2012), a high rate of respiratory opercular movement may result in an increase uptake of steroidal compounds such as estrogens. Fathead minnows were chosen for use in these studies as there are reports of their response to E1 exposure (Panter et al., 1998; Thorpe et al., 2007; Dammann et al., 2011) and tolerance to low dissolved oxygen (1 mg/L DO; Brungs, 1971; Nelson and Paetz, 1992).

1.3. Hypersalinization

The toxic effect of hypersalinization on freshwater organisms has been extensively documented (Adelman et al., 1976; Benbow and Merritt, 2005; Canada Council of Ministers of the Environment, 2011; Cañedo-Argüelles et al., 2013; Elphick et al., 2011; Evans, 1987; Peterson and Meador, 1994) and data indicate that environmental chloride concentrations are increasing temporally, especially in urban areas, as road salt applications increase (Corsi et al., 2015; Oliver et al., 1974). During times of low water flow in the summer and fall, chloride levels may exceed 800 mg/L, while normal freshwater levels fluctuate between 1 and 10 mg/L (New Hampshire Department of Environmental Sciences, 2013). Watersheds in metropolitan areas of the northeastern United States can become particularly saline in the winter months, with concentrations of up to 4629 mg chloride/L recorded (Kaushal et al., 2005). In addition to seasonal changes, it is forecasted that biogeochemical alterations associated with climate change will alter the salinity of aquatic systems (Moore et al., 1997). As the planet warms, an increase in severe rain events has been predicted (American Association for the Advancement of Science (AAAS), 2014; Hooper et al., 2013; Jeppesen et al., 2010; Noyes et al., 2009; Poff et al., 2002), as have rising sea levels causing saltwater intrusion into freshwater habitat (Murdoch et al., 2000) and drought (Taner et al., 2011; Whitehead et al., 2009; Williamson et al., 2008); all of which have the potential to alter the salinity of freshwater systems (Hooper et al., 2013; Jeppesen et al., 2010; Justic et al., 1996). More insidious than direct toxicity, perhaps, is the interplay between increasing chloride concentrations and a host of xenobiotic chemicals (Fortin et al., 2007; Heugens et al., 2001; Schiedek et al., 2007). For example, high salinity has been shown to increase the toxicity of chemicals, such as aldicarb, fenthion, and L-selenomethionine phorate, through formation of more toxic intermediates following saltwater acclimation in anadromous fish (Schlenk and Lavado, 2011). There is also evidence that salinity modulates the uptake of EE2 through changes in gill tissue morphology in male killifish, *Fundulus heteroclitus* (Blewett et al., 2012). Estrogens can act alone as a potent environmental endocrine disruptor (Colborn et al., 1993; Jobling et al., 1998; Schoenfuss et al., 2002; Schultz et al., 2013; Vajda et al., 2008), and are known for their relative ubiquity (Kolpin et al., 2002; Lee et al., 2011) especially in densely populated urban areas (Barber et al., 2011; Lee et al., 2011; Martinovic-Weigelt et al., 2013). The influence of salinity on estrogenic effects in fish has, to our knowledge, only been narrowly explored (Blewett et al., 2012).

Fathead minnows (*Pimephales promelas*) have a low chloride toxicity threshold (252 mg/L, chronic exposure) when compared with other freshwater organisms (Siegel, 2007). Increased salinity decreases oxygen solubility in the water, thus requiring changes in gill ventilation to maintain adequate oxygen uptake (Roberts, 2012). However, lipid-rich gill tissue is the primary site of entry for lipophilic estrogens into the bloodstream of teleosts, as exemplified by a threefold increase in the uptake of estrogen in male killifish maintained in 50% seawater when compared with freshwater (Blewett et al., 2012). An increase in salinity can also stress fish (Roberts, 2012; Siegel, 2007). A common stress-induced hormone, cortisol, has been shown to produce an enhancing effect on estrogen-induced vitellogenesis, upon intramuscular injection of hypophysectomized female Asian stinging catfish (*Heteropneustes fossilis*;10–20 μg cortisol/100 mg body weight; Hoar and Randall, 1988; Sundararaj et al., 1982).

1.4. Hypotheses

In the first set of exposure experiments (DO1; DO2), we examine the effect of dissolved oxygen (DO) concentration on biological endpoints commonly associated with estrogenic exposure. We hypothesized that hypoxia would increase the magnitude of E1 exposure effects. Estrone was used as a representative estrogenic compound due to its environmental relevance, as it is often found at higher frequency and higher concentrations than other estrogenic chemicals (Ankley et al., 2017, Dammann et al., 2011; Kolpin et al., 2002. A second set of experiments (SALT1; SALT2) tested the hypothesis that elevated sodium chloride concentrations exacerbate the effects of estrogen exposure. Male fathead minnows were exposed to environmentally common, but elevated, sodium chloride concentrations concurrently with exposure to environmentally common concentrations of E1. Fathead minnows were the appropriate test organisms as they have been shown to be suitable for investigating the effects of endocrine disrupting chemicals

(Ankley et al., 2009; Ankley and Johnson, 2004; Ankley and Villeneuve, 2006; Jensen et al., 2001; Watanabe et al., 2007), and previous reports have documented their response to estrone at environmental concentrations (reviewed in: Ankley et al., 2017). In addition, fathead minnows are broadly distributed in North America (Lee and Gilbert, 1980) and found in many freshwater system impacted by low DO and/or high sodium chloride concentrations. Lastly, the extensive use of fathead minnows in environmental toxicology provides numerous validated bioindicators of the effect of estrogenic exposure that can be utilized in the current study (Ankley et al., 2009; Ankley and Johnson, 2004; Ankley and Villeneuve, 2006; Jensen et al., 2001; Scholz and Mayer, 2008). These studies include the use of plasma vitellogenin as a bioindicator for estrogenic exposure (for example, Ankley et al., 2009; Vajda et al., 2008); secondary sex characteristics as an indicator of reduced male reproductive competence (for example Ankley et al., 2009); and the assessment of gonad and liver histology to examine maturity and pathologies in estrogen-exposed male fathead minnows (for example: Schoenfuss et al., 2016; Vajda et al., 2008).

2. Materials and methods

2.1. Exposure organisms

Adult male fathead minnows (~7 months old, mean length 66.5 mm and weight 35 g) were obtained from laboratory fish rearing facilities (DO experiments: Environmental Consulting & Testing, Superior, WI; SALT experiments: US EPA, Cincinnati, OH. Fish were acclimated in well water (the source for all experimental usage) for a minimum of 1 week prior to exposure. All fish were fed frozen brine shrimp (*Artemia* spp.) and frozen blood worms (*Glycera* spp.) ad libitum twice daily, and kept at a constant temperature of approximately 25°C with a 16 h light/8 h dark cycle. All fish maintenance was carried out in accordance with St. Cloud State University's IACUC guidelines (Protocol # 06-09).

2.1.1. DO experimental design

Two experiments were conducted at different times, each consisting of a 3 × 2 factorial design, with three E1 concentrations administered at two DO concentrations. Mature male fathead minnows were exposed to ethanol carrier control (no E1), low E1 or high E1 at low (no aeration) or a high (aerated) DO concentration for 21 days (Table 1). Unaerated well water was nearly anoxic, providing stable low DO concentrations, while high DO conditions were achieved through standard aeration of the tanks using air stones. Each of the resultant treatments was replicated in two aquaria, with 10 fish per tank ($n = 20$). Treatments were delivered using a flow-through design to aquaria in a modification of a published protocol (Schoenfuss et al., 2008).

Table 1
Mean measured concentrations of estrone (E1) ± standard deviation (sample size [n] in parenthesis) and dissolved oxygen (DO) in two male fathead minnow laboratory exposure experiments (DO1, DO2).

Treatment (abbreviation)	DO1	DO2
	mean ± stand. dev. (n)	mean ± stand. dev. (n)
Control	no E1 detected (4)	no E1 detected (4)
Low DO	1.7 ± 0.30 mg DO/L (106)	1.6 ± 0.48 mg DO/L (126)
High DO	9.5 ± 0.36 mg DO/L (106)	9.1 ± 0.55 mg DO/L (126)
Low E1	29 ± 43 ng E1/L (4)	51 ± 12.6 ng E1/L (4)
Low DO	1.3 ± 0.30 mg DO/L (106)	2.0 ± 0.66 mg DO/L (126)
High DO	9.4 ± 0.40 mg DO/L (106)	8.9 ± 0.62 mg DO/L (126)
High E1	232 ± 316 ng E1/L (4)	390 ± 83.3 ng E1/L (4)
Low DO	1.6 ± 0.42 mg DO/L (106)	1.8 ± 0.33 mg DO/L (126)
High DO	9.3 ± 0.52 mg DO/L (106)	8.8 ± 0.56 mg DO/L (126)

2.1.2. SALT experimental design

The experiment consisted of 5 treatment groups: low salt EtOH solvent control with no E1, low salt low E1, high salt low E1, high salt low E1, and high salt high E1. A high salt only treatment was not included due to facility limitations. The same original stock of fish were used for both experiments, but the fish were two months older at the start of the second SALT (2) experiment. Each of the five treatments was replicated in two aquaria, with 10 fish per tank ($n = 20$). The exposure period was limited to 10 days in order to avoid mortality associated with prolonged hypersalinity.

2.1.3. Exposure chemicals

Estrone (CAS # 53-16-7, Reference Grade (99.3% purity), Sigma-Aldrich, St. Louis, MO) exposure solutions were prepared daily from aliquots of a concentrated E1 solution in ethanol (VWR, Radnor, PA). Analysis of stock estrone solutions in ethanol diluted to ~100 pg/μL and analyzed by LCMSMS detected no estriol, 17α- or β-estradiol (detection limits 0.5 pg/ul on column). Controls received ethanol equivalent to that in E1 treatments (<0.001% EtOH). Aliquots were stored at 4 °C until use. Each day of the exposure, one treatment-specific aliquot was mixed with 10 L of well water (dedicated supply of ground water) in amber glass dosing bottles. In order to ensure chemical dosing was consistent and "settling out of dose" was avoided, dosing bottles were maintained on stir plates. Stainless steel tubing and a stainless steel mixing chamber with a Cole-Parmer (Vernon Hills, IL) Masterflex 7523-40 peristaltic pump delivered the solutions at a nominal rate of 7 mL/min. Well water or well water with added NaCl was also fed into the mixing chamber at a rate of 200 mL/min to achieve the final treatment concentration. Mixture was achieved by allowing the combined solution to fall over two barrier-walls within the mixing tank. The final solution was delivered directly to four aquaria via tubing extending from the bottom of the tank. Water exchange rate was approximately seven exchanges/aquarium/day.

2.1.4. Water chemistry

Physico-chemical parameters (temperature, conductivity, total dissolved solids, salinity, DO, pH, and oxidation reduction potential) of each aquarium were recorded each morning (YSI Model 556 MPS, YSI Environmental, Yellow Springs, OH). Additional DO and temperature readings were recorded during the afternoon and evening. Total water hardness, free chlorine, total chlorine and alkalinity were measured periodically using AquaChek 5-in-1 Water Quality Test Strips (Hach Company, Loveland, CO).

For DO1 and DO2, water samples were taken from each treatment tank on exposure days 5,10, 15, 20, and on the day of dissection (120 mL each). For SALT 1, samples were taken on exposure days 5 and 10, with two additional samples added for SALT 2 (duplicate samples on day 5 and 10) to increase confidence in assayed values. Estrone concentrations were determined by LC-MS^2 using previously published methods (Dammann et al., 2011). Deuterated E1 standard (d_4, CDN, Quebec, CA) was added to samples prior to solid phase extraction to yield nominal concentrations of 40 or 20 pg/μL on resuspension at either ~35 (low E1 dose) to ~700 (high dose E1) fold original sample volume, resulting in E1 concentrations from ~10 to 40 pg/μL on column, respectively (limit of quantitation, 1 pg/μL on column). These adjustments were made to compensate for the previously observed ion enhancing effect seen in highly concentrated samples. Ion concentrations were analyzed using EPA method 200.7 (Martin et al., 1992) and 300.0 (Pfaff, 1993).

2.2. Biological endpoints

2.2.1. Plasma vitellogenin analysis

Blood was drawn from the severed caudal vasculature using a heparinized capillary tube after fish were anesthetized in 0.1% MS-222 (Argent Laboratories, Redmond, WA). Blood was centrifuged at 8050 x *g*

for 8 min at 4 °C to obtain plasma that was stored at −80 °C prior to analysis.

Plasma vitellogenin (VTG) concentrations (μg/mL) were quantified via competitive antibody-capture ELISA using an 8-point serial dilution standard (4.8 μg/mL to 0.0375 μg/mL). Detailed methods of the assay are described in Shappell et al. (2010).

2.2.2. Blood glucose

Using 1 μL of whole blood, a TRUEbalance Blood Glucose Monitor (Moore Medical, Farmington, CT) was used to quantify blood glucose concentration (detection range of 20 to 600 mg/dL) using instrument specific single-use measuring strip.

2.2.3. Hematocrit

Blood was taken via the caudal artery using 75 mm heparinized capillary tubes (ClearCRIT Plastic Capillary Tubes, Separation Technology Inc., Sanford, FL). Capillary tubes were sealed (Critoseal, Oxford Labware, St. Louis, MO), centrifuged (HERMLE Z200A, Labnet International Inc., Woodbridge, NJ) for 8 min at 8050 ×*g*, and hematocrit (HCT) measured (Spiracrit Micro-Hematocrit Tube Reader, Clay-Adams Inc., New York, NY).

Organosomatic indices

Prior to dissection, fish were weighed and measured for length to calculate body condition factor (BCF = body weight/total length^3, Fulton, 1904). To calculate hepatosomatic index (HSI = liver weight/whole body weight × 100) and gonadosomatic index (GSI = gonad weight/whole body weight × 100), liver and gonads were excised and immediately weighed (0.001 g precision, Mettler Toledo AG245, Columbus, OH).

2.2.4. Secondary sex characteristics

Prior to dissection, a visual assessment of the male sexual characteristics of individual fish was performed. Tubercles, dorsal pad and banded coloration were given a separate rating of 1, 2 or 3 based on prominence (1 being the least prominent, 3 being the most prominent) (Smith, 1978). The three values were analyzed separately, and as a single composite value as a method for comparing morphological sexual maturity between treatments.

2.2.5. Histopathology

Liver and gonads were excised from fish and placed in histological cassettes in 10% buffered formalin for at least 24 h. Dehydration, tissue preparation, and paraffin embedment were as in previously prescribed procedures (Carson, 1997). Small samples (~9 mm^2 surface area) of liver and gonad were sectioned at 5 μm (Olympus Cut 4055 Microtome, Olympus America Inc., Center Valley, PA). A standard haematoxylin and eosin counter stain was used on both tissues (Carson, 1997; Gabe, 1976).

Liver tissues were graded on a scale of 1 to 4 based on cellular vacuolization. A grade of 1 indicates that no vacuoles were visible under a 40× magnification, a grade of 2 indicates that <25% of the visible tissue was occupied by vacuoles, a grade of 3 indicates that 25–50% of the visible tissue was occupied by vacuoles, and a grade of 4 indicates that the majority of the visible tissue consisted of vacuoles.

Testes were assessed based on the proportion of sperm cells at various stages of development (spermatogonia, spermatocytes, spermatids, and spermatozoa) present in the visible tissue. Percentage values (totaling 100%) were assigned to individual cell types within each sample. The overall maturity of the sample was calculated as:

Gonad maturity rating (GMR) = $((\%_{\text{spermatogonia}}) + (\%_{\text{spermatocytes}} \times 2) + (\%_{\text{spermatids}} \times 3) + (\%_{\text{spermatozoa}} \times 4)) / 100$.

All histological assessment of tissues was performed without knowledge of treatment to eliminate observational bias.

2.2.6. Statistical analysis

The variables HCT, glucose, VTG, GMR, BCF, liver vacuolization, GSI and HSI from the DO experiments were analyzed using two-way analysis of variance (ANOVA) to test for effects of E1, DO and their interaction. In the ANOVAs, E1 was considered to have 3 levels: control (0), 29 ng/L or 232 ng/L. In the SALT experiments, there were 5 groups, no control high sodium chloride included. The same variables measured in the DO experiments were analyzed using a one-way ANOVA. A priori contrasts were included within the ANOVAs to test for effects of E1, salt and their interaction on data from the 4 treated groups (E1 = low or high estrone; and Salt = low or high NaCl). Tukey's multiple comparison tests were used for all pairwise comparisons. Glucose and VTG values were log-transformed prior to analysis. Transformed data were not found to violate assumptions of parametric distribution. In all experiments, tank was considered the sampling unit. Sex characteristics (individual and summed) and liver vacuolization were analyzed for treatment effects using Chi-square statistics, with fish as the individual sampling unit. The summed measure of SSC, were categorized low (scores of 3–6) or high (scores of 7–9) for analysis. In the DO experiments, the number of fish with scores of 3 was very low, so scores were combined and evaluated as either 1′s or 2′s and 3′s. SAS version 9.4 was used for all analyses, significance indicated at $P = 0.05$.

3. Results and discussion

3.1. Dissolved oxygen/estrone exposure experiments

3.1.1. Measured concentrations of estrone and dissolved oxygen

Dissolved oxygen concentrations were similar for the corresponding DO treatments in both experiments (~1.7 and ~9.2 mg/L for low and high, respectively; Table 1). Because source water containing E1 (low or high) was split between aquaria exposed to low or high DO, E1 concentrations did not differ by DO treatment. In DO1, E1 treatments averaged 29 (low E1) and 232 (high E1) ng/L (Table 1). The ratio of high to low E1 concentrations were ~8 fold in both DO1 and DO2, but measured mean E1 concentrations in DO2 were higher (51 and 390 ng/L, respectively). The variability in measured estrone concentrations throughout the exposure period was greater than expected. One explanation could be related to the affinity of estrogens for organic material, rather than aqueous matrices, which potentiates loss upon delivery to tanks. In attempts to minimize this effect, supply lines were routinely changed out throughout the experimental period. The variability seen may have also be a result of mechanical discrepancies, resulting in altered flow rates. Because estrone concentrations were different in DO1 and DO2, the experiments were analyzed separately.

3.1.2. Treatment effects

Percent survival (Table 2, two tanks per treatment) ranged from 75% to 100%. Treatment effects within an experiment could not be tested as $n = 2$, though interestingly, the tanks with the lowest survival in both DO1 and DO2 were within the high E1, low DO treatment (data not shown). The F or Chi Square values, degrees of freedom, and P values for main effects and interactions are given at the top of Table 2 a (DO1) and 2b (DO2). Of the nine parameters measured, differences were observed in four of those in DO1, and three in DO2. The parameters in which treatment effects were seen varied by experiment, with the exception of VTG, which was significant ($P \leq 0.0002$). Estrone treatment at either low or high concentrations increased VTG concentrations above those in control fish (SI Fig. 1). It is logical to assume that enhanced respiratory rates under low DO conditions would result in an increase in the actual E1 dose absorbed by the fish, resulting in higher rates of VTG production. Though not statistically different, the mean VTG for low E1/low DO treatment were higher than low E1/high DO in both experiments. The experimenters observed behavioral changes related to low DO treatment of both increased respiratory rates and increased presence of fish at the site of water entry into the aquaria. Both of

Table 2
Survival, hematological, and morphological parameters (mean ± standard error) in male fathead minnows exposed to estrone (E1) at two dissolved oxygen (DO) concentrations in two exposure experiments (**a.** DO1, **b.** DO2). BCF = body condition factor; GSI = gonadosomatic index; HSI = hepatosomatic index; SSC = secondary sex characteristics; GMR = testis maturity; VTG = vitellogenin; HCT = hematocrit. Variable sample size for individual parameters reflect mortality or insufficient tissue for measurement or analysis in surviving fish. Bold print indicates significant differences (P ≤ 0.05, variable-specific differences reported in text, statistical analyses as described in methods).

a. **DO1**	Survival	**BCF**	GSI	**HSI**	**SSC**	GMR	Liver vacuolization	Glucose	**VTG**	HCT
ANOVA	NA	$F_{E1\ 2,6} = 10.2$ **P = 0.01** $F_{DO\ 1,6} = 3.8$ P = 0.10	$F_{E1\ 2,6} = 0.63$ P = 0.57 $F_{DO\ 1,6} = 0.44$ P = 0.53	$F_{E1\ 2,6} = 14.5$ **P = 0.005** $F_{DO\ 1,6} = 1.6$ P = 0.25	$Chi\ Sq_{E1\ 2} = 3.7$ P = 0.16 $Chi\ Sq_{DO\ 1} = 4.3$ **P = 0.04**	$F_{E1\ 2,6} = 0.64$ P = 0.56 $F_{DO\ 1,6} = 0.05$ P = 0.84	$Chi\ Sq_{E1\ 2} = 4.6$ P = 0.10 $Chi\ Sq_{DO\ 1} =$ 0.6 P = 0.45	$F_{E1\ 2,6} = 1.1$ P = 0.38 $F_{DO\ 1,6} = 0.02$ P = 0.88	$F_{E1\ 2,6} = 117$ **P < 0.0001** $F_{DO\ 1,6} = 5.2$ P = 0.06	$F_{E1\ 2,6} = 2.3$ P = 0.18 $F_{DO\ 1,6} = 1.3$ P = 0.29
Treatment Effect		$E1 \times DO\ _{2,6} =$ 1.8 P = 0.25	$E1 \times DO\ _{2,6} =$ 0.62 P = 0.57	$E1 \times DO\ _{2,6} =$ 1.2 P = 0.37	$ChiSq_{E1\ \times\ DO\ 2} = 1.8$ P = 0.41	$E1 \times DO\ _{2,6} =$ 2.8 P = 0.14	$ChiSq_{E1\ x\ DO\ 2} =$ 0.4 P = 0.80	E1 x DO $_{2,6} =$ 0.88 P = 0.46	E1 x DO $_{2,6} =$ 2.4 P = 0.17	E1 x DO $_{2,6} =$ 2.0 P = 0.22
Control Low DO	80%	1.10 ± 0.02	1.30 ± 0.34	1.14 ± 0.14	4.56 ± 0.53	3.36 ± 0.10	2.28 ± 0.40	158 ± 60	2.1 ± 0.65	50.1 ± 3.20
Control High DO	90%	1.16 ± 0.02	1.76 ± 0.34	1.20 ± 0.14	5.06 ± 0.53	3.65 ± 0.10	2.50 ± 0.37	190 ± 4.7	1.4 ± 0.14	51.0 ± 3.20
Low E1 Low DO	95%	1.02 ± 0.02	1.68 ± 0.34	1.03 ± 0.14	3.74 ± 0.44	3.65 ± 0.10	2.00 ± 0.35	109 ± 19	149 ± 4.4	58.8 ± 3.20
Low E1 High DO	100%	1.07 ± 0.02	1.43 ± 0.34	0.91 ± 0.14	5.40 ± 0.52	3.58 ± 0.10	2.00 ± 0.38	95 ± 10.8	78.8 ± 35.4	48.5 ± 3.20
High E1 Low DO	75%	1.08 ± 0.02	1.04 ± 0.34	1.91 ± 0.14	3.60 ± 0.49	3.63 ± 0.10	2.57 ± 0.43	111 ± 37	145 ± 7.0	46.6 ± 3.20
High E1 High DO	95%	1.06 ± 0.02	1.38 ± 0.34	1.53 ± 0.14	4.06 ± 0.49	3.46 ± 0.10	3.21 ± 0.48	171 ± 66	127 ± 56.2	46.9 ± 3.20

b. **DO2**	Survival	BCF	**GSI**	HSI	SSC	GMR	Liver vacuolization	Glucose	**VTG**	**HCT**
ANOVA	NA	$F_{E1\ 2,6} = 0.41$ P = 0.68 $F_{DO\ 1,6} = 1.0$ P = 0.35	$F_{E1\ 2,6} = 6.8$ **P = 0.03** $F_{DO\ 1,6} = 2.5$ P = 0.16	$F_{E1\ 2,6} = 4.5$ P = 0.06 $F_{DO\ 1,6} = 0.02$ P = 0.88	$Chi\ Sq_{E1\ 2} = 0.27$ P = 0.87 $Chi\ Sq_{DO\ 1} = 0.07$ P = 0.79	$F_{E1\ 2,6} = 0.05$ P = 0.95 $F_{DO\ 1,6} = 0.11$ P = 0.75	$Chi\ Sq_{E1\ 2} = 0.05$ P = 0.98 $Chi\ Sq_{DO\ 1} = 0.27$ P = 0.60	$F_{E1\ 2,6} = 0.85$ P = 0.47 $F_{DO\ 1,6} = 4.3$ P = 0.08	$F_{E1\ 2,6} = 48.2$ **P = 0.0002** $F_{DO\ 1,6} = 0.00$ P = 0.96	$F_{E1\ 2,6} = 33.5$ **P = 0.0006** $F_{DO\ 1,6} = 2.0$ P = 0.21
Treatment Effect		$E1 \times DO\ _{2,6} =$ 0.02 P = 0.98	$E1 \times DO\ _{2,6} =$ 2.2 P = 0.19	$E1 \times DO\ _{2,6} =$ 2.5 P = 0.16	$ChiSq_{E1\ \times\ DO\ 2} =$ 0.77 P = 0.68	$E1 \times DO\ _{2,6} =$ 0.20 P = 0.83	$ChiSq_{E1\ \times\ DO\ 2} =$ 0.23 P = 0.89	$E1 \times DO\ _{2,6} =$ 1.0 P = 0.41	E1 x DO $_{2,6} =$ 0.10 P = 0.91	E1 x DO $_{2,6} =$ 0.40 P = 0.69
Control Low DO	90%	1.19 ± 0.04	1.07 ± 0.12	1.41 ± 0.16	4.17 ± 0.48	3.66 ± 0.12	2.14 ± 0.39	208 ± 7.8	1.4 ± 0.16	61.1 ± 2.6
Control High DO	100%	1.16 ± 0.04	1.31 ± 0.12	1.2 ± 0.16	4.75 ± 0.49	3.71 ± 0.12	2.54 ± 0.48	121 ± 21.6	1.1 ± 0.14	62.0 ± 2.6
Low E1 Low DO	85%	1.15 ± 0.04	1.20 ± 0.12	1.63 ± 0.16	4.35 ± 0.51	3.67 ± 0.12	2.20 ± 0.47	159 ± 18.6	156 ± 102	55.8 ± 2.6
Low E1 High DO	100%	1.12 ± 0.04	1.08 ± 0.12	1.38 ± 0.16	4.10 ± 0.45	3.63 ± 0.12	2.38 ± 0.43	159 ± 3.8	63.4 ± 11.6	58.3 ± 2.6
High E1 Low DO	80%	1.19 ± 0.04	0.63 ± 0.12	0.81 ± 0.16	4.44 ± 0.53	3.70 ± 0.12	2.25 ± 0.43	171 ± 7.3	86.6 ± 49.0	38.4 ± 2.6
High E1 High DO	90%	1.14 ± 0.04[b]	0.97 ± 0.12	1.21 ± 0.16	4.44 ± 0.50	3.59 ± 0.12	2.23 ± 0.41	124 ± 21[c]	86.9 ± 47.9	43.9 ± 2.6

these behaviors are consistent with literature reports, resulting in individual fish being exposed to environmental endocrine disruptors at higher than predicted concentrations. Vitellogenin response is known to plateau with higher doses of estrogen, and therefore not surprising that no increase with low DO was observed at high E1 doses (Korte et al., 2000; Schmid et al., 2002; Thorpe et al., 2007).

Maturity of the testis (GMR), liver vacuolization, and blood glucose were not affected by either E1 or DO treatment in either experiment, and only minor differences in survival were seen ($n = 2$ tanks per treatment, Table 2). However, several variables were altered by E1 exposure (BCF and HSI in DO1; with GSI and HCT in DO2). In DO1, relative liver size (HSI) was increased by high E1 treatment ($\bar{x} = 1.72 \pm 0.10$ SE) relative to either control ($\bar{x} = 1.17$, P 0.02) or low E1 ($\bar{x} = 0.97$, P 0.005) exposure, irrespective of DO concentrations. In contrast, in DO2 relative liver size in the high E1 treatment ($\bar{x} = 1.01 \pm 0.16$ SE) tended to be less than the low E1 treatment ($\bar{x} = 1.50$, P 0.06), but not the control ($\bar{x} =$ 1.30). Similar variability in HSI of male fathead minnows has been observed previously in response to E1 (Dammann et al., 2011). A partial explanation of the variability found in HSI could be differences in body weights of fish within one aquaria, independent of E1 treatment, as a consequence of established dominance among fish (Ivanova et al., 2016)). Relative testis size (GSI) was significantly depressed in DO2 by high E1 treatments ($\bar{x} = 0.80 \pm 0.08$ SE) relative to control ($\bar{x} = 1.19$, P 0.03) though not statistically lower than low E1 ($\bar{x} = 1.14$, P0.06). This effect was not observed in DO1, possibly due to the lower E1 in DO1 for the high E1 treatments.

Statistically, low DO concentrations decreased SSC in DO1, where low DO $\bar{x} = 3.9 \pm 0.28$ SE and high DO $\bar{x} = 4.8 \pm 0.29$ SE (P 0.04, Table 2a). Though not statistically different, in DO1, mean SSC for high E1 treatments (low and high DO) were lower than their respective control counterparts (Table 2a). While SSC are discussed as the sum of all three morphological parameters (tubercle, dorsal pad, and banding coloration), they were also analyzed separately as reported in the supplemental information (SI Fig. 2–4). In both DO experiments, fish exposed to high E1 concentrations (232 and 390 ng E1/L) at low DO received the lowest possible tubercle score (1) in blind scoring (SI Fig. 2). With low DO, control fish had higher tubercle scores than either E1 treatment in DO1 (P = 0.01), while in DO2, tubercle scores for both control and low E1 were higher than those exposed to high E1 (P = 0.04). Similarly, in DO1 but not DO2, high DO control and low E1 treatments resulted in higher tubercle scores than those exposed to high E1 treatments (P = 0.02). Dorsal pad scores differed only in DO2, and surprisingly, scores were lower for control than either E1 treatment group (P = 0.02) when DO was low (SI Fig. 3). Color differences were found

only in DO1, with high DO and low E1 treatment resulting in higher color scores than for either control or high E1 exposed to high DO ($P = 0.02$, SI Fig. 4). The variable responses of individual SSCs with treatments suggests that summing SSCs may alter perceived treatment responses. Decreased SSC with E1 exposure were previously reported for male fathead minnows (Dammann et al., 2011). Declining SSC scores likely the result of androgen suppression in the HPG axis. Male reproductive performance is regulated by the hypothalamus-pituitary-gonadal axis with increasing androgen concentrations resulting in greater male aggressiveness and increased expression of SSCs (Smith, 1978). Estrogenic exposure can reduce plasma androgen concentrations resulting in a decline of SSCs (Trudeau, 1997). Similarly, exposure to other environmental stressors, such as hypoxia will increase production of corticosteroids and reduction of reproductive hormones, including androgens (Pottinger et al., 1996).

The effect of E1 on HCT varied by experiment (Fig. S1). Hematocrit declined significantly in high E1 treatments in DO2 ($P < 0.001$), but not DO1. The mean HCT in high E1-treated fish (41% ± 1.8 SE at 390 ng/L E1) was significantly lower than both control (61.5% $P < 0.001$) and low E1-treated fish (57.1% P 0.002, at 51 ng/L,) in DO2. Again the higher E1 concentrations in DO2 (390 ng E1/L vs 232 ng E1/L in DO1) may have resulted in the differing results between DO1 and DO2. Renal toxicity due to glomerular vitellogenin accumulation at very high concentrations of E2 has been observed in male summer flounder (*Paralichthys dentatus*; Folmar et al., 2001). If kidney damage resulted from the 390 ng E1/L treatment, production of erythropoietin in the kidneys (not measured) may have been affected, in turn reducing red blood cell production. Immature sturgeon (*Acipenser stellatus*) were reported to have lower HCT when injected intraperitoneally with E2 (Falahatkar et al., 2014), as did female brook trout (*Salvelinus fontinalis*) after consuming E2-treated feed for one month (Schafhauser-Smith and Benfey, 2003). Others have reported conflicting results. Osachoff et al. (2014) found juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ethinylestradiol (EE2) or E2 (0.5 and 1 μg/L) for four days had no change in HCT values. A 20% increase in HCT (not statistically significant) was observed in female red grouper receiving i.p. injections of E2, every other day for 9 days (Ng et al., 1984).

3.2. Sodium chloride/estrone exposure experiments

3.2.1. Measured concentrations of estrone and sodium chloride

Mean E1 concentrations in the first experiment (SALT1) were 18 and 193 ng/L respectively for the low and high treatments (Table 3). Estrone concentrations did not differ between the respective low and high sodium chloride treatments as the common source water for both sodium chloride treatments already contained E1 at the final concentration. Mean E1 concentrations were lower in SALT2 (7 and 85 ng/L respectively for the low and high E1 treatments), and therefore experiments were analyzed separately. Salt concentrations were similar between the two experiments for the respective low (~28 mg/L Na^+, ~96 mg/L Cl^-) and high (~54 mg/L Na^+, ~133 mg/L Cl^-) NaCl treatments (Table 3).

Table 3
Mean measured concentrations ± standard deviation (sample size [n] in parenthesis) of estrone (E1), sodium (Na^+), and chloride (Cl^-) in two male fathead minnow laboratory exposure experiments (SALT1 and 2).

Treatment (abbreviation)	SALT 1	SALT 2
	mean ± stand. dev. (n)	mean ± stand. dev. (n)
	no E1 detected	no E1 detected
Control	27 ± 0.3 mg/L Na^+ (3)	29 ± 0.1 mg/L Na^+ (3)
(well)	94 ± 0.1 mg/L Cl^- (3)	98 ± 0.3 mg/L Cl^- (3)
Low E1	18 ng E1/L (2)	7 ± 1.8 ng E1/L (4)
Low salt	27 ± 0.3 mg/L Na^+ (3)	29 ± 0.1 mg/L Na^+ (3)
	94 ± 0.1 mg/L Cl^- (3)	98 ± 0.2 mg/L Cl^- (3)
High salt	59 ± 4.1 mg/L Na^+ (3)	45 ± 2.9 mg/L Na^+ (3)
	141 ± 4.6 mg/L Cl^- (3)	120 ± 5.5 mg/L Cl^- (3)
High E1	193 ng E1/L (2)	85 ± 32.3 ng E1/L (4)
Low salt	27 ± 0.3 mg/L Na^+ (3)	29 ± 0.1 mg/L Na^+ (3)
	94 ± 0.1 mg/L Cl^- (3)	98 ± 0.2 mg/L Cl (3)
High salt	59 ± 4.1 mg/L Na^+ (3)	52 ± 7.9 mg/L Na^+ (3)
	141 ± 4.6 mg/L Cl^- (3)	131 ± 12.1 mg/L Cl^- (3)

3.2.2. Treatment effects

Survival was not adversely affected by any of the treatments in either experiment (mean of two tanks per treatment, Table 4), with the lowest survival for any one tank of 80% (control, data not shown). This rate of mortality is not out of the ordinary when all males are maintained in one tank. Indices altered by treatment in both SALT1 and 2 were BCF and VTG. Overall, mean body condition factor tended to be lower for fish in high E1 treatments (Table 4a and b), though higher variability of BCF was present in SALT2. High E1 at either sodium chloride concentration resulted in lower BCF (~1.02) than high SALT low E1 ($\bar{x} = 1.25 \pm 0.04$, $P \leq 0.05$) in SALT2.

Similar effects of E1 on VTG, the primary indicator of estrogenic exposure, were observed in SALT1 and 2 (SI Fig.5). Vitellogenin concentrations were not significantly elevated above control in any low E1 treatment in either SALT experiment (Table 4). This is most likely a reflection of the low E1 concentrations (18 and 7 ng/L for SALT1 and SALT2, respectively), as Dammann et al. (2011) found VTG increased when male fathead minnows were exposed to ≥31 ng E1/L. High E1 exposure (193 ng/L in SALT1) resulted in VTG concentrations significantly higher than control or low E1 treatments ($P < 0.0001$) independent of sodium chloride concentrations. In SALT2 (Table 4b), "high E1 fish" were exposed to lower E1 concentrations ($\bar{x} = 85$ ng/L), but VTG was still elevated relative to control and low E1 treatments ($P \leq 0.04$), though comparison of low E1 to high E1 at low sodium chloride conditions failed to meet significance ($P = 0.06$).

While blood glucose was not affected by treatment in SALT1, in SALT2 an interaction between E1 and sodium chloride concentration was observed ($P = 0.03$). Blood glucose was lower in the low E1 high SALT group ($\bar{x} = 93.4 \pm 5.8$ SE mg/mL) than in the high E1 high SALT group ($\bar{x} = 139.4 \pm 0.6$ SE, $P = 0.03$). Similarly, independent of salt treatment, high E1 treatment resulted in higher blood glucose than low E1 or control groups (140 mg/mL vs 105 or 108 mg/mL, respectively, $P = 0.02$). The highest mean values observed in the high E1 treatment corresponded with the group of fish with the highest VTG concentrations. Median glucose concentrations for control, low E1 and high E1 were 92, 94, and 148 mg/dL, respectively (SI Fig.5). It is unclear why higher variability in blood glucose was seen in SALT1 as no methodological changes occurred. Speculation for the cause of variability could include variations in blood collection times due to the variable experience of personnel taking samples, potentially masking significant treatment differences. While literature reports of the effect of E1 on blood glucose in fish is lacking, available literature on fish are divided on the effect of E2 on plasma glucose. Washburn et al. (1992, 1993) found both male and female rainbow trout implanted with E2 exhibited lower plasma glucose. Similarly, rainbow trout exposed for four days to 0.5 and 1 μg E2/L also had decreased plasma glucose concentrations (Osachoff et al., 2014). In contrast, juvenile sturgeon receiving i.p. injections of E2 were found to have higher glucose concentrations (Falahatkar et al., 2014), as did juvenile salmon (*Salmo salar*) exposed to 2 μg E2/L for 21 days (Lerner et al., 2007). Species, route of administration, or dose (pharmacological in the case of the literature cited) may have resulted in the differences reported.

The rest of the indices examined (GSI, HSI, GMR, liver vacuolization, HCT, and SSC) remained unaltered across treatments (exception, GMR for SALT2 showed an effect of E1 treatment without differences in pairwise comparisons). In addition, no differences were found in SSC when characteristics were analyzed individually (prominence of tubercle, dorsal pad, or banding coloration prominence) in SALT1 or 2 (SI

Table 4
Survival, hematological, and morphological parameters (mean ± standard error) in male fathead minnows exposed to estrone (E1) at two sodium chloride (SALT) concentrations in two exposure experiments (**a.** SALT1, **b.** SALT2). Abbreviations defined in Table 2. Bold print indicates significant differences ($P \leq 0.05$, variable-specific differences reported in text, statistical analyses as described in methods).

a

SALT1	Survival	**BCF**	GSI	HSI	SSC	GMR	Liver vacuolization	glucose	**VTG**	HCT
ANOVA (group)	NA	$F_{4,23} = 6.5$ **P = 0.001**	$F_{4,23} = 0.9$ P = 0.48	$F_{4,23} = 1.46$ P = 0.25	Chi Sq.df_4 = 1.84 P = 0.76	$F_{4,23} = 1.34$ P = 0.29	Chi Sq. df_4 = 8.64 P = 0.08	$F_{4,15} = 0.31$ P = 0.87	$F_{4,23} = 65.4$ **P < 0.0001**	$F_{4,23} = 0.42$ P = 0.79
Treatment effect (P value)		**E1 P = 0.006** Salt 0.86 E1 × Salt 0.27	E1 P = 0.66 Salt 0.22 E1 × Salt 0.76	E1 P = 0.20 Salt 0.92 E1 × Salt 0.47	E1 P = 0.82 Salt 0.32 E1 × Salt 0.56	E1 P = 0.93 Salt 0.22 E1 × Salt 0.08	E1 P = 0.13 Salt 0.09 E1 × Salt 0.98	E1 P = 0.92 Salt 0.29 E1 × Salt 0.96	**E1 P < 0.0001** Salt 0.46 E1 × Salt 0.19	E1 P = 0.51 Salt 0.42 E1 x Salt 0.64
Control (well, low salt)	88%	1.24 ± 0.03^{ac}	0.92 ± 0.12	1.38 ± 0.09	6.75 ± 0.65	3.40 ± 0.17	1.56 ± 0.31	111 ± 41	4.8 ± 1.81^{a}	57.2 ± 4.20
Low E1 low salt	95%	1.25 ± 0.02^{a}	1.16 ± 0.08	1.20 ± 0.07	6.81 ± 0.46	3.65 ± 0.12	2.13 ± 0.26	78 ± 26	7.4 ± 0.99^{a}	54.5 ± 2.96
Low E1 high salt	95%	1.22 ± 0.03^{ab}	1.00 ± 0.12	1.26 ± 0.09	5.94 ± 0.61	3.20 ± 0.17	1.62 ± 0.32	109 ± 25	8.5 ± 0.86^{a}	59.2 ± 4.20
High E1 low salt	100%	1.13 ± 0.02^{b}	1.08 ± 0.08	1.16 ± 0.07	6.34 ± 0.44	3.40 ± 0.12	2.68 ± 0.29	84 ± 30	150 ± 25^{b}	58.6 ± 2.96
High E1 high salt	98%	1.15 ± 0.03^{bc}	0.98 ± 0.12	1.09 ± 0.09	6.12 ± 0.62	3.48 ± 0.17	2.06 ± 0.36	105 ± 26	118 ± 25^{b}	59.9 ± 4.20

b

SALT2	Survival	**BCF**	GSI	HSI	SSC	GMR	Liver vacuolization	**Glucose**	**VTG**	HCT
ANOVA	NA	$F_{4,5} = 6.8$ **P = 0.03**	$F_{4,5} = 1.5$ P = 0.34	$F_{4,5} = 1.62$ P = 0.30	Chi Sq.df_4 = 6.30 P = 0.18	$F_{4,5} = 3.23$ P = 0.12	Chi Sq. df_4 = 5.04 P = 0.28	$F_{4,5} = 5.68$ **P = 0.04**	$F_{4,5} = 15.6$ **P = 0.005**	$F_{4,5} = 1.55$ P = 0.32
Treatment effect (P value)		**E1 P = 0.0**1 Salt 0.14 E1 × Salt 0.17	E1 P = 0.12 Salt 0.33 E1 × Salt 0.45	E1 P = 0.26 Salt 0.35 E1 × Salt 0.11	E1 P = 0.11 Salt 0.97 E1 × Salt 0.11	**E1 P = 0.03** Salt 0.43 E1 × Salt 0.58	E1 P = 0.06 Salt 0.09 E1 × Salt 0.84	**E1 P = 0.02** Salt 0.32 **E1 × Salt 0.03**	**E1 P = 0.001** Salt 0.23 E1 × Salt 0.21	E1 P = 0.16 Salt 0.66 E1 × Salt 0.16
Control (well, low salt)	89%	1.20 ± 0.04^{abc}	0.92 ± 0.15	1.51 ± 0.12	6.44 ± 0.63	3.45 ± 0.15	1.50 ± 0.32	107.6 ± 6.5^{abc}	3.2 ± 0.21^{a}	57.5 ± 6.76
Low E1 low salt	94%	1.12 ± 0.04^{abc}	1.19 ± 0.15	1.28 ± 0.12	5.63 ± 0.59	3.43 ± 0.15	1.94 ± 0.37	115.8 ± 1.0^{abc}	3.6 ± 0.10^{ab}	64.8 ± 6.76
Low E1 high salt	94%	1.25 ± 0.04^{ac}	1.16 ± 0.15	1.64 ± 0.12	6.71 ± 0.69	3.39 ± 0.15	2.39 ± 0.45	93.4 ± 5.8^{ac}	3.6 ± 0.56^{ab}	72.7 ± 6.76
High E1 low salt	94%	1.02 ± 0.04^{b}	1.04 ± 0.15	1.67 ± 0.12	5.63 ± 0.59	3.07 ± 0.15	1.37 ± 0.30	140.2 ± 5.6^{ac}	42.9 ± 12.1^{bc}	64.6 ± 6.76
High E1 high salt	89%	1.03 ± 0.04^{b}	0.76 ± 0.15	1.56 ± 0.12	4.75 ± 0.54	2.86 ± 0.15	1.56 ± 0.33	139.4 ± 0.6^{b}	74.0 ± 18.8^{c}	50.4 ± 6.76

Fig. S6). Increasing E1 concentrations were previously reported to decrease SSC in 21-day exposure experiments with fathead minnows (15 to 54, and 10 to 46 ng/L E1; Dammann et al., 2011) for 21 days. The shortened exposure period used in the SALT experiments (10 days) may explain the differences in results.

Sodium chloride concentration was not found to alter any other biomarkers assessed in the current study, with the exception of an interaction with E1 on blood glucose (SALT2). However, the chloride concentrations tested fall well below the United States Environmental Protection Agency's chronic water quality criteria threshold of 230 mg/L (U.S. Environmental Protection Agency, 1988). Therefore, this subject should be revisited, especially in light of recent evidence that chloride concentrations in surface waters often persist at concentrations much higher than those tested in this SALT1 or SALT2 (Corsi et al., 2015). In future studies, it would be advisable to increase total chloride concentration to at least 230 mg/L, approaching the chronic toxicity threshold for *P. promelas* (252 mg/L) in order to examine how the organism responds to E1 with a higher degree of chloride-induced osmoregulatory stress (Adelman et al., 1976; Evans, 1987; Siegel, 2007).

4. Conclusions

The current study sought to determine whether environmental conditions (DO and sodium chloride concentration) alter the biological effects of E1 exposure in adult male fathead minnows. Reduced DO concentrations were hypothesized to result in physiological and anatomical changes in respiration and gill morphology in fish, leading to greater E1 uptake and exacerbated effects of E1 exposure. Low DO was found to result in an increased VTG response to lower E1 concentrations, most likely the response to resultant "higher exposure dose" due to increased respiratory rates and increased presence of fish at the site of water entry into the aquariaWhile we predicted that increased added sodium chloride would increase E1 uptake, thereby increasing biological consequences of E1 exposure, the concentrations tested fail to alter observed responses. This was most likely a result of the low concentrations used.

While the authors acknowledge the relevance of LOECs for broad environmental applications has been questioned (Landis and Chapman, 2011), the data from the current study is useful in clarifying a laboratory LOEC for E1 in fathead minnows. In all four exposure experiments (SALT1 &2, DO1 &2), aqueous concentrations of up to 18 ng E1/L had no significant effect in elevating VTG concentrations, regardless of salinity or DO alteration. In contrast, VTG concentrations were significantly elevated in treatments receiving at least 29 ng E1/L. These findings are consistent with the published literature and suggest that lowest observed effects concentrations (LOEC) for VTG elevation in male fathead minnows are between 18 and 29 ng E1/L (Dammann et al., 2011; Panter et al., 1998). Interestingly, the LOEC for all other endpoints was also 29 ng E1/L, potentially indicating that vitellogenesis is related, possibly directly, to the other effects observed (SSC, DO1, Table 2a, GSI, DO2,

Table 2b) and concentration-dependent changes in HSI (DO1 and DO2, Table 2a & b). Changes in SSC and GSI, while not observed in all experiments, are consistent with previous studies that indicate anatomical feminization following estrogen exposure (Dammann et al., 2011; Shappell et al., 2010).

The results of the current study evidence the variability of response to E1, even within the confines of controlled laboratory conditions, and despite experimental conditions and data that met the criteria for reporting and evaluating ecotoxicity data (Moermond et al., 2015), with the exception of a high salt treatment, precluded for inclusion by facility limitation. The ambiguity of data may, in part reflect our model's failure to represent field situations in many ways, but perhaps most importantly it fails to account for the presences of females. Within a tank of 10 males, dominance is established, which has been shown to impact variables measured (Ivanova et al., 2016), and potentially explaining the lack of consistent statistical differences in treatments. Similarly, small, perhaps transient alterations in environmental conditions may not affect the expression of biomarkers of estrogenic endocrine disruption, even though physiological changes as a result of environmental change are well documented in the literature. However, if predicted environmental events are exacerbated by anthropogenic factors such as urbanized land use or climate change, fish sensitivity to estrogenic endocrine disrupting compounds may have to be re-evaluated.

Disclosure

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. USDA is an equal opportunity provider and employer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.07.251.

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